

Aminopeptidases in Mycelium and Growth Medium of *Streptomyces rimosus* Strains

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Received: November 11, 2008

Accepted: May 27, 2009

Summary

Aminopeptidases (APs) of the same substrate specificities have been detected in the mycelia and culture filtrate of *Streptomyces rimosus*. To compare extracellular and intracellular prolyl, leucyl and arginyl AP, dynamics of their biosynthesis, excretion and localization were analyzed during submerged cultivation of two *S. rimosus* strains, T55 and ZGL3, in several media. AP activity in mycelia reached maximum in the stationary phase, and decreased to different extent at a later stage. The accumulation of APs, except prolyl aminopeptidase (ProAP), in the culture filtrate followed the growth of bacteria and decreased later on, when peptide-rich medium was used. When *S. rimosus* was grown in glucose-rich medium, the accumulation of APs in the medium started at the late log phase and continued to the end of cultivation, due to cell lysis. The combined addition of calcium and ammonium salts to tryptone soy broth increased the AP activity in *S. rimosus* ZGL3 culture filtrates up to two times. The AP intracellular activity was significantly higher compared to its intercellular activity (2 to 24 times). Mycelium/medium AP activity ratio decreased with the age of the culture, its change being dependent on the *S. rimosus* strain, growth medium composition and AP specificity. Leucyl AP (LeuAP) was the most prone to be released from the mycelium, suggesting that part of the enzyme could be excreted by active transport. Determination of AP distribution within cell compartments has confirmed that the three APs are intracellular enzymes residing in cytosol, but also suggested their partial association with cytoplasmic membrane.

Key words: prolyl, leucyl and arginyl aminopeptidases; localization; *Streptomyces rimosus*

Introduction

Streptomyces, filamentous Gram-positive bacteria, are known to synthesize numerous endo- and exopeptidases, which can be localized within or outside the cell. Time course of biosynthesis, accumulation, excretion and activity of each one might be influenced by the composition of cultivation medium and other growth conditions (1–5). The amounts and consequently the ratios of various peptidases can change with the culture age, as shown by electrophoretic patterns of enzymes from actinomycetes *Frankia* sp. (6). In addition, the corre-

lation between mycelium morphology and endo- and aminopeptidase activities was also observed (7). *Streptomyces rimosus* synthesizes a number of peptidases and several of them have been isolated and characterized (8–13). In search for proline-specific peptidases of *S. rimosus* grown under conditions for oxytetracycline production, activity of prolyl aminopeptidase (ProAP) was found in its mycelium and culture filtrate. Although ProAP draws attention due to its possible application for collagen degradation and debittering of milk products, there is little data on this type of enzyme from streptomycetes. Within *Streptomyces* genus, ProAP was described

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This paper was presented at the Congress of Croatian Society of Biochemistry and Molecular Biology in Osijek, Croatia, September 17–20, 2008

as an extracellular enzyme of *S. plicatus* (14) and as an intracellular one in *S. lividans* (15), while in the related genus *Frankia*, the presence of ProAP within and outside the cell was reported. Sodium chloride tolerant ProAP was isolated from culture filtrate of a 4-day-old *S. aureofaciens* culture and well characterized (16). This raised a question of the number and nature of the enzymes responsible for the detected ProAP activity in *S. rimosus* culture. The changes of intra- and extracellular hydrolytic activities, characteristic of prolyl, and the previously described leucyl and arginyl aminopeptidases (11,12) during cultivation of two *S. rimosus* strains in different media are examined. Possible differentiation parameters, molecular mass and isoelectric point of the corresponding ProAPs are also determined.

Materials and Methods

Microorganisms

Two *Streptomyces rimosus* strains were used, *S. rimosus* T55, provided by Krka Pharmaceutical Works, Novo mesto, Slovenia, and *S. rimosus* ZGL3, the kind gift of Dr Jasenka Pigac, Ruđer Bošković Institute, Zagreb, Croatia.

Media and conditions for microorganism growth

S. rimosus was cultivated in trichloroacetic acid (TCA) medium, tryptone soy broth (TSB) and sporulation medium (SM). TCA medium (17) contained (in g): bacto-trypton 2.0, yeast extract 2.0, $\text{MgSO}_4 \cdot 6\text{H}_2\text{O}$ 0.5, D(+)-glucose 10.0 and distilled water up to 1 L. TSB contained (in g): pancreatic digest of casein 17.0, papaic digest of soybean meal 3.0, glucose 2.5, NaCl 5.0, K_2HPO_4 2.5, with or without $(\text{NH}_4)_2\text{SO}_4$ 2.0 and CaCO_3 7.0, and distilled water up to 1 L. SM contained (in g): soybean meal 20.0, manitol 20.0, with or without $(\text{NH}_4)_2\text{SO}_4$ 2.0 and CaCO_3 7.0, and distilled water up to 1 L. Before sterilization, pH value was adjusted to 7.0. Media were autoclaved for 20 min at 120 °C.

Erlenmeyer flasks (500 mL) containing 80 mL of medium were inoculated with 4 mL of 24-hour-old *S. rimosus* culture obtained from spores, and cultivated on a rotary shaker at 250 rpm (Tehtnica RVI-403, Železniki, Slovenia) at 28 and 30 °C for 96 and 120 h in TSB/SM and TCA medium, respectively.

The growth and formation of the microorganism

S. rimosus growth was followed by determining the increase of mycelium dry mass. Every 24 h, 5-mL aliquots were centrifuged and the collected mycelia were dried at 110 °C for 3 h, until constant mass was obtained. The pH of the supernatants was also measured.

To monitor mycelium morphological changes, the samples were taken at 24-hour intervals, stained according to Gram, and inspected by microscope (Zeiss-Axiovert 35, Oberkochen, Germany).

Culture volume was maintained constant by replacing the removed samples with the same volume of the culture from the spare Erlenmeyer flasks.

Dynamics of aminopeptidase accumulation in culture filtrate and mycelium

Culture filtrate

Aliquots (5 mL) of *S. rimosus* culture were centrifuged for 15 min at 500×g at room temperature. Supernatants were removed, clarified by centrifugation at 15 700×g for 5 min and used for aminopeptidase assay. Combined sediments were dried and weighted.

Mycelium

For the analysis of AP activity in mycelium, 2-mL culture aliquots were taken and centrifuged at 5150×g for 10 min at room temperature. Supernatants were decanted and mycelia washed by resuspending in 1.5 mL of saline, pH=7.0, and sedimenting at 15 700×g for 10 min. The collected mycelium was resuspended in 0.5 mL of 10^{-2} M sodium phosphate buffer, pH=7.0, containing 0.1 M NaCl and 4 mg/mL of lysozyme (approx. 88 000 U/mL, Serva, Heidelberg, Germany) and incubated in a shaker for 40 min at 30 °C. The mycelium was additionally treated with ultrasound 3 times for 2 min with cooling (20 kC/s, MSE 100 W disintegrator, London, UK). Aliquots of the obtained suspension served for the determination of AP activity.

Distribution and location of APs in *S. rimosus* culture

For this analysis, *S. rimosus* (ZGL3) culture grown for 48 hours in TCA medium was used. Culture supernatants and mycelium were obtained and treated as already described. Activity of APs and marker enzymes was determined in the culture medium, in the suspension of washed mycelium and in the suspension of disrupted mycelium.

For determination of AP distribution within the mycelium, biomass collected at 1500×g was washed with 0.3 mol/L of sucrose and resuspended in buffer P for protoplast formation (18). One aliquot was used for protoplast formation and enzyme location following the procedure of Aparicio *et al.* (19) (treatment with 4 mg/mL of lysozyme, centrifugation, lysis in water, centrifugation), while another one passed through the same procedure without the lysozyme and served as control.

Enzyme assay

Aminopeptidase activity was measured at 37 °C with Arg-, Leu- and Pro-2-naphthylamide (2NA) as substrates (Serva, Heidelberg, Germany) using colorimetric assay for determination of liberated 2-naphthylamine (20). The standard reaction mixture of 1 mL contained: 50 mM Tris-HCl buffer, pH=7.5, 8.8 µM substrate (plus 1 mM CaCl_2 with Leu-2NA) and an enzyme sample. The reaction was started with the addition of substrate to the equilibrated enzyme solution. After incubation for 15 to 30 min in a water bath shaker, the reaction was stopped by the addition of 0.2 mL of Fast Blue B salt (FBB) reagent containing: 1.5 mg/mL of dye in 2.1 M sodium-acetate buffer, pH=4.2, with 10 % Tween 20 (Serva, Heidelberg, Germany). The developed colour was measured at 530 nm against the control, where the enzyme was added after the addition of FBB reagent. Standard curve was constructed with the known amounts of 2-naphthyl-

amine. Enzyme unit was defined as the amount of enzyme that hydrolyzed 1 μ mol of substrate per min.

Activity of glucose-6-phosphate dehydrogenase (G6P-DH), used as intracellular marker enzyme for cytosol, was determined following the procedure given by the substrate manufacturer (Boehringer, Mannheim, Germany). Activity of DNase, used as extracellular and periplasmic enzyme marker, was determined as previously described (21).

Molecular mass and isoelectric point determination

Molecular mass and isoelectric point of aminopeptidases were determined using non-fractionated growth medium and mycelium extract.

Molecular mass was determined by polyacrylamide gel electrophoresis under native conditions on 8–25 % gradient PhastGel plates (PhastSystem Pharmacia, Uppsala, Sweden), and by gel filtration on Sephacryl S-200 superfine column (0.9 \times 51.5 cm) in 50 mM sodium phosphate buffer, pH=7.0, containing 0.2 M NaCl, at a flow rate of 6 mL/h.

The isoelectric point of APs was determined by isoelectric focusing on PhastGel plates with pH=3–9 and pH=4.0–6.5, following the instructions of the manufacturer (Pharmacia, Uppsala, Sweden), and also on 245 \times 110 \times 1 mm plates with 2.2 % (by mass per volume) ampholines, pH=3.5–9.5 or 4.0–6.5, using Multiphor II 2117 (LKB, Bromma, Sweden) cooled with running water. Gels were prefocused for 30–40 min at 450 V and 30 mA for plates with pH=3.5–9.5, and at 600 V and 25 mA for plates with pH=4.0–6.5. Samples of 15 μ L with approx. 100 μ g of proteins were applied to filter paper squares

(0.5 \times 1 cm) laid on gel surface. After 30–45 min at 700 V and 25 mA, the filter paper was removed and focusing continued at 1200 V and 30 mA for plates with pH=3.5–9.5, and at 1200 V and 25 mA for plates with pH=4.0–6.5. One lane contained pI standards (broad pI or low pI, pH=4.0–6.5, Pharmacia, Uppsala, Sweden). The position of aminopeptidases on gels was determined by cutting 1-mm gel slices and assaying the enzyme activity. The lane with standards was stained with Coomassie Brilliant Blue.

Samples were concentrated by ultrafiltration using Diaflo PM10 and PM30 membranes (Centricon, Amicon, Oosterhout, the Netherlands).

Results and Discussion

S. rimosus growth, AP biosynthesis and distribution

Cultures of both examined strains of *S. rimosus*, T55 and ZGL3, grown in any of the examined media had aminopeptidases splitting Pro-, Arg- and Leu-2NA in the culture filtrates and within mycelia. To test the strain dependence and the influence of the growth medium composition and culture age on the amount of synthesized APs (estimated as their activity), and on the ratio of biomass-bound and excreted or released enzyme, both culture filtrates and mycelia were analyzed during *S. rimosus* cultivation. As illustrated in Figs. 1 and 2 *S. rimosus* growth curves were typical for submerged batch cultures of bacteria, having defined exponential phase, stationary phase and cell lysis at its end. Between strains T55 and ZGL3, only slight differences in growth dynamics were observed. Strain ZGL3 had shorter log phase in

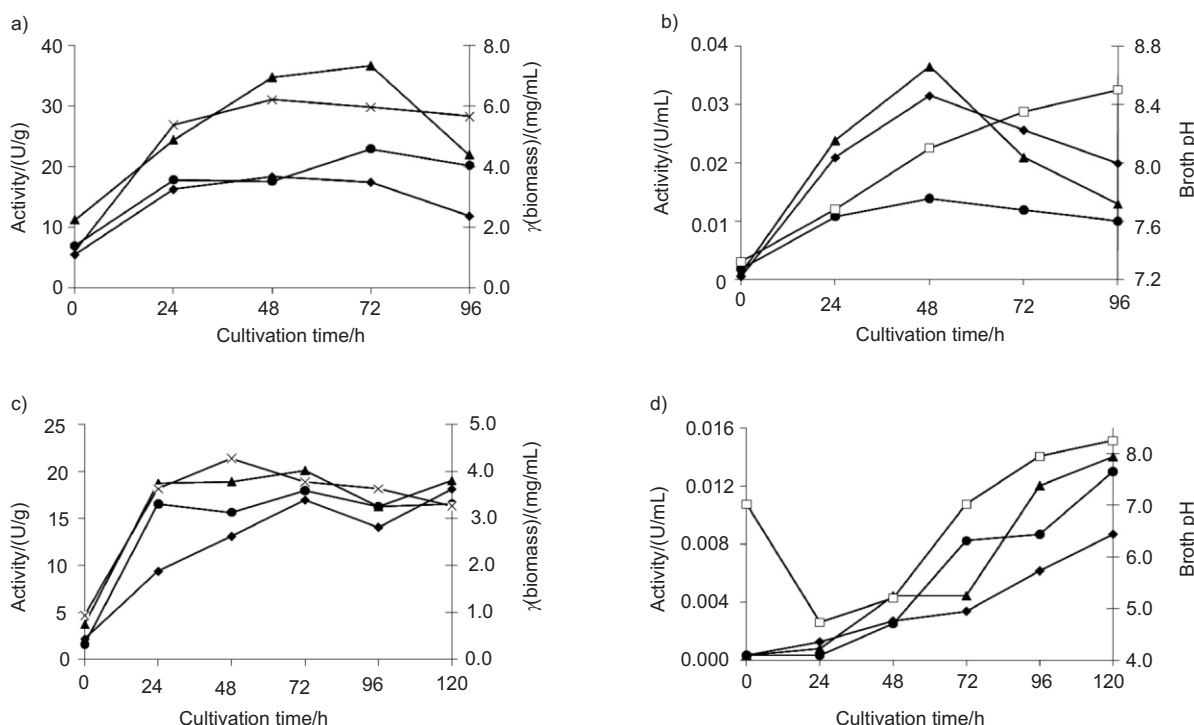


Fig. 1. *S. rimosus* T55 growth (x- biomass; -□- broth pH) and aminopeptidase activity (●- ProAP; -▲- ArgAP; -◆- LeuAP) in bio-mass (a, c) and in culture filtrate (b, d) during cultivation in TSB (a, b) and in TCA medium (c, d). Results are mean values of three cultivations

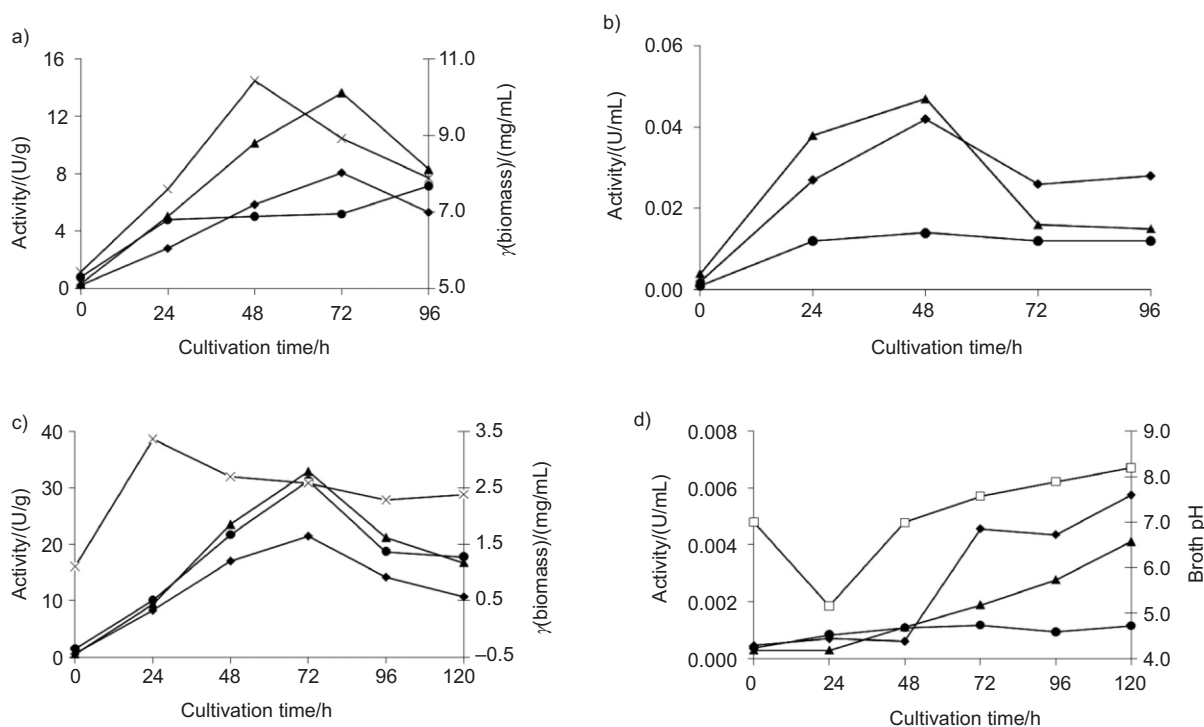


Fig. 2. *S. rimosus* ZGL3 growth (—x— biomass; —□— broth pH) and aminopeptidase activity (—●— ProAP; —▲— ArgAP; —◆— LeuAP) in biomass (a, c) and in culture filtrate (b, d) during cultivation in TSB (a, b) and in TCA medium (c, d). Results are mean values of three cultivations

TCA medium and larger biomass loss in both, TCA and TSB media, while T55 strain had extended stationary phase. Microscopic examination (Fig. 3) reflected mycelium changes. Filamentous Gram-positive pellets prevailing in young culture until stationary phase progressively turned into fragmented hyphae, which started to stain as Gram-negative bacteria at 72 h due to cell wall changes caused by aging. Strain ZGL3 started aging slightly earlier. *S. rimosus* does not produce spores in liquid media.

Aminopeptidase accumulation presented in Figs. 1 and 2 had different dynamics in the mycelium and culture broth, and it was affected by medium composition. All cellular AP activities reached maximum at stationary phase, and decreased to a different extent towards the end of cultivation. ProAP was the most stable enzyme. The highest activity of ProAP was obtained by strain ZGL3 grown in the glucose-rich TCA medium containing yeast extract. In all cases, total activity in the mycelium was several times larger than in the corresponding culture filtrate (Table 1). Their ratio was highest at the log phase, declining later on, for both strains and growth media. AP biosynthesis, parallel to the bacterial growth, and their retention within mycelium even at stationary phase indicate their primary importance for the intracellular catabolism of *S. rimosus*. In cases when peptidases are needed for degradation of substrates outside the cell, the amounts of retained enzymes are smaller than the amounts of the excreted ones. Thus in *Serratia marcescens* culture only 1–10 % of total trypsin-like peptidase remained within the cells (22).

The release of *S. rimosus* APs into the liquid medium was affected by its composition. In peptide-richer

TSB medium, ArgAP and LeuAP extracellular activity followed *S. rimosus* growth to the middle of stationary phase and then decreased. ProAP activity accumulated more slowly but did not change much during stationary phase. In glucose-rich TCA medium, AP secretion started at the late log phase and continued to the end of cultivation. Maximal ArgAP and LeuAP activities exceeded that of ProAP, except when T55 strain was grown in TCA medium. The amount of ArgAP and LeuAP in the culture filtrates at the end of growth in the two media increased relative to the amount in the mycelium, LeuAP being more prone to be released. The behaviour of ProAP did not follow that trend (Table 1). Parallel *S. rimosus* growth and ArgAP and LeuAP activity curves in TSB might suggest active excretion process. However, this could not be said for the growth in TCA medium and for the ProAP. According to the presented findings, the APs are mainly released from the autolyzed *S. rimosus* mycelium grown on the examined media.

There is very little published data on the dynamics of biosynthesis and on intra- and extracellular activities of aminopeptidases in streptomycetes. Aphale and Strohl (15) reported LeuAP and ProAP activities in mycelium and in growth broth of *S. lividans* 1326, a 120-hour-old culture in TSB, but without data on their amounts. Nevertheless, from their specific activities (ProAP higher in the cell, LeuAP in the culture filtrate), it could be concluded that ProAP is dominantly intracellular, whereas LeuAP is of intra- and extracellular type. Certain differences between ProAP and LeuAP of *S. rimosus* are indicated by changes of their intra- and extracellular activity ratios during stationary phase. If enzymes were in the medium only because of cell lysis, their parallel release

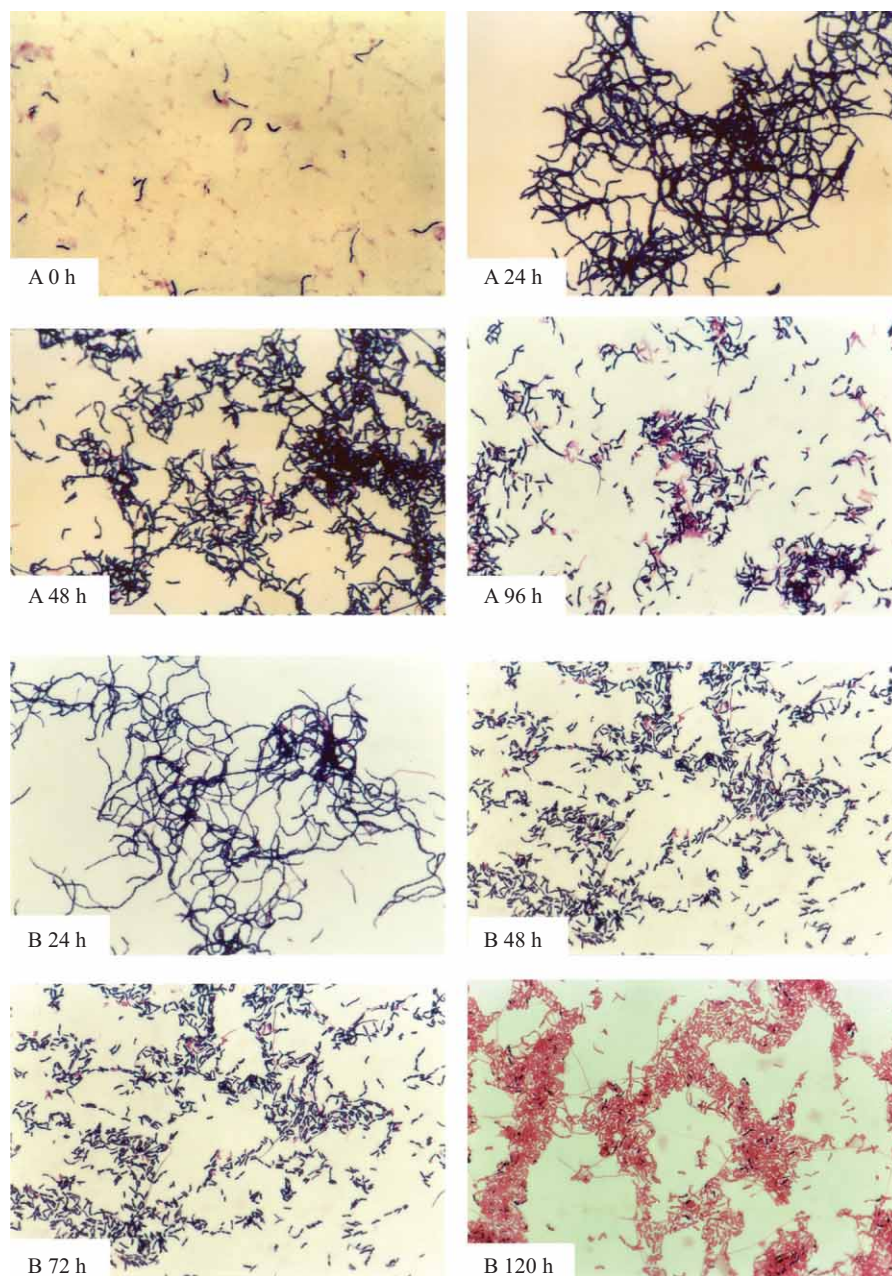


Fig. 3. Morphology of *S. rimosus* mycelium (A) strain T55 and (B) strain ZGL3 grown in TCA medium (Gram staining, magnification 6300×)

Table 1. Percentage of total AP activity located in the mycelium of *S. rimosus* cultures

Substrate	AP activity* / %			
	TCA culture		TSB culture	
Strain T55	72 h	120 h	72 h	96 h
Pro-2NA	89	81	92	92
Arg-2NA	94	81	91	90
Leu-2NA	95	87	80	77
Strain ZGL3				
Pro-2NA	92	86	82	85
Arg-2NA	90	78	89	83
Leu-2NA	82	64	72	63

*Results are mean values of three *S. rimosus* cultivations

would be expected. Relatively more intensive excretion of LeuAP suggests the existence of enzymes with different destination. In closely related genus *Frankia*, intra- and extracellular APs were compared by electrophoretic analysis (6). Unlike in *S. rimosus* culture, ProAP activity in *Frankia* reached its maximum before the end of logarithmic phase, and decreased later on to the undetectable level, keeping its intracellular level unchanged. This could be a consequence of a very low excretion and high susceptibility to proteolytic degradation in the medium. LeuAP continued to be excreted to the end of cultivation. In culture filtrates of *S. plicatus* and *S. aureofaciens*, from which extracellular ProAPs were purified (14,16), the enzyme activities were of the same order of magnitude as ProAP activity in *S. rimosus* broth. Although in-

tracellular concentrations are not available for comparison, this might suggest that isolated ProAPs belong to the intracellular type of enzymes, as does *S. rimosus* ProAP. Purified intracellular ProAPs of some other bacteria were shown to have high molecular mass polymeric structure (23–26).

There are numerous reports revealing that calcium and ammonium as constituents of nutrient media affect peptidase synthesis and excretion by bacteria, few of them dealing with aminopeptidases. The influence of Ca^{2+} and inorganic nitrogen source added to the medium on the amount of APs in *S. rimosus* ZGL3 culture filtrate was examined (Table 2). The effect of these additions was not identical in all cases. Calcium ions slightly suppressed ArgAP and LeuAP release and did not affect ProAP in peptide-rich TSB, but they did decrease its activity in the sporulation medium, which contains manitol instead of glucose. Ammonium ions raised the levels of AP activity in both media to various extents, with concomitant decrease of activity ratio in mycelium and culture filtrate, suggesting the stimulation of excretion. Combined addition of Ca^{2+} and NH_4^+ significantly elevated examined AP activities in TSB and suppressed ProAP and LeuAP activity in SM. Similar effect was obtained with both strains. It is obvious that the response of *S. rimosus* to the added CaCO_3 and/or $(\text{NH}_4)_2\text{SO}_4$ depends on other nutrients available for its growth. Unequal effects of NH_4^+ as nitrogen source on the peptidases from streptomyces illustrate the data on endopeptidases. Thus Lalue and Molinari (1) showed that low concentrations of $(\text{NH}_4)_2\text{SO}_4$ in the growth medium containing yeast ex-

tract stimulated *S. aureofaciens* to synthesize and secrete caseinolytic peptidase, while higher concentrations had inhibitory effect. At the same time, CaCO_3 slightly suppressed protease secretion. Bascarán *et al.* (3) reported that NH_4Cl , when added to the medium with amino acids, decreased extracellular production of metalloprotease in the culture of *Streptomyces clavuligerus*. $(\text{NH}_4)_2\text{SO}_4$ was the optimal nitrogen source for the production of endopeptidase secreted by *S. rimosus* (5). Its presence was stimulating even when added to the medium with organic nitrogen source. CaCO_3 also increased levels of extracellular proteolytic activity. Influence of temperature stress and peptone starvation in the presence of ammonium on intracellular LeuAP was studied in *Streptomyces hygroscopicus* (2), showing increased synthesis of the enzyme. In general, the effect of a single nutrient was valid for a particular medium and growth conditions. Thus the addition of $(\text{NH}_4)_2\text{SO}_4$ and CaCO_3 to the TSB could be used to enrich *S. rimosus* culture filtrate if it were chosen for isolation of ProAP.

Aminopeptidase localization within mycelium

Bacterial ProAPs associated with cells could be membrane bound (27,28), periplasmic (29), or cytoplasmic (15,30), where they could be part of a larger proteolytic complex (31,32). Since the major part of total AP activities of *S. rimosus* culture was found in the biomass, their location within mycelium was investigated. Sequential extraction of APs and determination of marker enzyme activities in extracts and cells of 48-hour-old ZGL3 culture in TCA medium were performed (Table 3). The collected mycelium contained 96 % of ProAP, 90 % of ArgAP, 77 % of LeuAP, 100 % of cytosol marker G6P-DH, and 1 % of extracellular DNase activity of the whole culture. When intact in a suspension, the mycelium did not hydrolyze any of the offered AP substrates, showing that the examined enzymes are not associated with the surface of cell wall. Lysozyme treatment without osmotic cell lysis caused significant release of DNase, which is in accordance with its periplasmic location while within mycelium (19,21). Among APs, LeuAP was relatively easiest to extract, but total amounts of released APs were negligible, excluding those localized in the periplasm. Although protoplast formation was not extensive, enzyme release from the mycelium treated with lysozyme and H_2O , but not with H_2O alone, revealed that APs are located within cytosolic compartment of the cell. Slight differences in behaviour from that of G6P-DH indicate that partially they might be weakly associated with cy-

Table 2. Influence of the addition of ammonium and calcium ions to the growth medium on relative amount of APs in *S. rimosus* ZGL3 culture filtrates

Medium*	Activity**/%		
	Pro-2NA	Arg-2NA	Leu-2NA
TSB with added:	100	100	100
Ca^{2+}	105	90	79
NH_4^+	104	135	121
Ca^{2+} and NH_4^+	177	200	204
SM with added:	100	100	100
Ca^{2+}	51	76	82
NH_4^+	187	121	109
Ca^{2+} and NH_4^+	37	117	55

*Cultivation time in TSB 96 h, in SM 120 h

**Results are mean values of two cultivations

Table 3. Localization of APs in mycelium of *S. rimosus**

Enzyme released from	Percentage of total activity in mycelium				
	ProAP	ArgAP	LeuAP	G6P-DH	DNase
Mycelium in buffer P	0.5	0.9	2.2	0.0	29.8
Collected mycelium in H_2O	2.0	2.8	2.5	0.0	2.6
Mycelium in buffer P and lysozyme	0.8	1.6	3.0	1.0	78.9
Mycelium treated with lysozyme and H_2O	39.4	42.3	38.7	0.4	21.9
Remained mycelium disintegrated with ultrasound	63.8	53.2	55.1	95.7	12.8
Total	104.0	107.1	97.7	97.1	113.6

**S. rimosus* ZGL3 48-hour-old culture in TCA medium

toplasmic membrane. The obtained results confirm that the examined APs are intracellular, mainly cytosolic enzymes that leave mycelium after its lysis. They do not exclude the possibility that part of the synthesized enzymes, primarily LeuAP, is directly released into the culture medium (33).

To test the similarity or difference between APs in the mycelium and culture filtrate, their molecular mass and isoelectric point were determined. Gel filtration revealed two proline-specific APs within mycelium, one having molecular mass around 213 kDa, similar to the enzyme from the filtrate (227 kDa), and the other having molecular mass of 90 kDa (data not shown). By isoelectric focusing of mycelium extract, two bands with ProAP-specific activity were also obtained, one at pH=4.9 and the other at pH=4.6 (data not shown). Molecular mass of ArgAP and LeuAP was of the same order of magnitude as the smaller ProAP. By its size and pI (at pH=4.4), ArgAP corresponds to the previously isolated intracellular ArgAP from the same microorganism (12). The enzyme splitting Leu-2NA differs from the previously isolated extracellular LeuAP of *S. rimosus* (11), which might be differently processed or another enzyme. By its size, the larger ProAP resembles the hexameric ProAP with protomer of 42 kDa, isolated from the filtrate of *S. aureofaciens* culture (16), whereas the presence of the smaller one indicates a possibility of another ProAP in *S. rimosus*.

Conclusion

Besides other peptidases, *S. rimosus* strains T55 and ZGL3 produce aminopeptidases that hydrolyze peptide bonds of proline, arginine and leucine. The major part of all three enzymes remains within mycelium, and the minor one is released to the medium, mainly due to the cell lysis, which classifies them as intracellular enzymes. Their quantity, dynamics of synthesis and excretion can be altered by strain selection, basal medium composition, addition of calcium and ammonium salts, and length of cultivation. General rules could not be set, the levels and distribution of enzymes are rather valid for a particular overall set of growth conditions. The observed differences in enzyme behaviour might rise from the synthesis of more than one type of AP with the same substrate specificity. There is strong indication that *S. rimosus* has two intracellular ProAPs, and it remains to be elucidated whether they are different enzymes or just two forms of the same enzyme.

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